

Tissue distribution of rat glutathione transferase subunit 7, a hepatoma marker

Sally E. PEMBLE,* John B. TAYLOR and Brian KETTERER

Cancer Research Campaign Molecular Toxicology Group, Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, Mortimer Street, London W1P 7PN, U.K.

Polyadenylated RNA isolated from *NN*-dimethyl-4-aminoazobenzene-induced rat hepatoma was used to prepare a cDNA library in λ gt10. Full-length clones complementary to mRNA coding for glutathione transferase subunit 7 were isolated and one of these clones (pGSTR7) was fully characterized. In Northern blot analysis, mRNA hybridizing to 32 P-labelled pGSTR7 was found in poly(A)-containing RNA isolated from seven normal rat tissues but not from testis and liver. A similar hybridizing mRNA species was also detected in human placental mRNA. The same probe, used in a Southern blot analysis of genomic DNA, suggests the presence of a multigene family in the rat.

INTRODUCTION

The glutathione (GSH) transferases are dimeric enzymes which have an important role in the inactivation of highly reactive electrophilic compounds and endogenous hydroperoxides hazardous to the well-being of the cell. In addition the enzymes have important transport and biosynthetic roles, for example in the hepatic uptake of bilirubin and the synthesis of leukotriene C. The isoenzymes also show marked tissue-specific distribution presumed to result from the different roles and substrates encountered in each tissue. The subunit compositions of the GSH transferases of both man and rat have been extensively studied. In both species, subunits have been allotted to families on the criteria of their amino acid composition, peptide maps, *N*-terminal sequences, subunit-subunit association, and activities toward a variety of substrates (for review see Mannervik, 1985). Using these criteria, subunits 1 and 2 and subunits 3 and 4 of the rat enzymes were reported to be the products of multiple genes which fall into two gene families (Beale *et al.*, 1982, 1983). The application of cDNA cloning and sequencing techniques has confirmed that at least two gene families, namely the subunit 1/2 family and subunit 3/4 family, exist for the rat enzymes (Pickett *et al.*, 1984; Lai *et al.*, 1984; Taylor *et al.*, 1984; Telakowski-Hopkins *et al.*, 1985; Ding *et al.*, 1985; Lai *et al.*, 1986; Rothkopf *et al.*, 1986). Of at least four subunits known to exist, only one (subunit 7) has been identified by means of a cDNA sequence, synthesized from mRNA isolated from an AAF-induced rat hepatoma (Suguoka *et al.*, 1985).

GSH transferase 7-7 is of especial interest since it was observed to occur at high level in hepatoma but is apparently absent from normal liver (Kitahara *et al.*, 1984; Meyer *et al.*, 1985; Satoh *et al.*, 1985) and was identified with a protein previously described as a hepatoma marker (Farber, 1984). GSH transferase 7-7 is also noteworthy for its high GSH peroxidase activity towards fatty acid and thymine hydroperoxides (Meyer *et al.*, 1985; Tan *et al.*, 1986). The amino acid sequence of subunit 7, deduced from the cDNA sequence

(Suguoka *et al.*, 1985), has some homology to the subunit 1/2 family. However, on the basis of *N*-terminal amino acid sequence and enzymic properties, subunit 7 has been assigned to a third class of GSH transferases (Mannervik *et al.*, 1985).

In this paper we report the characterization of several subunit 7 cDNA clones synthesized from mRNA isolated from a DAB-induced rat hepatoma. We have used one of these clones as a probe to examine the distribution of hybridizing RNA species in poly(A)-containing RNA isolated from DAB-induced hepatoma and nine normal tissues of the rat. We also demonstrate the presence of a hybridizing RNA species in poly(A)-containing RNA isolated from human placenta and lymphocytes. In addition we show by Southern blot analysis of rat genomic DNA that there is likely to be more than one genomic sequence highly complementary to subunit 7 mRNA and that subunit 7 is an example of a third gene family of the GSH transferases of the rat.

MATERIALS AND METHODS

Materials

*Eco*RI, *Bam*HI, *Pst*I, *Bgl*II, *Ava*II, and *Sma*I restriction endonucleases, ribonuclease H and polynucleotide kinase were obtained from Bethesda Research Laboratories through Gibco Life Technologies, Paisley, U.K. Phosphorylated *Eco*RI octameric linkers, *Escherichia coli* DNA ligase and *Eco*RI methylase were obtained from New England Biolabs, Bishops Cleeve, U.K. *Hind*III restriction nuclease and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim (BCL), Lewes, U.K. T_4 DNA ligase, deoxynucleotides and the cloning vectors pUC13, M13mp10 and M13mp11 were purchased from Pharmacia, Milton Keynes, U.K. *E. coli* DNA polymerase 1, T_4 DNA polymerase, Hybond-N blotting membrane and all radioisotopes were obtained from Amersham International, Amersham, U.K. S_1 nuclease was obtained from Sigma, Poole, U.K. Ultrogel AcA34 was purchased from L.K.B., Croydon, U.K. All other materials,

Abbreviations used: DAB, *NN*-dimethyl-4-aminoazobenzene; AAF, *N*-acetyl-2-aminofluorene.

* To whom reprint requests should be addressed.

enzymes, chemicals and solvents were obtained from sources described previously (Pemble *et al.*, 1986, and references therein).

Animals

All normal rat tissues were obtained from male Wistar rats weighing 180–200 g inbred at the Courtauld Institute of Biochemistry. Hepatomas were induced in rat livers as described in Meyer *et al.* (1985).

Preparation of poly(A)-containing RNA

Total RNA was isolated from 5 g of rat hepatoma or normal rat tissue and from full term human placenta and human lymphocytes by a modification of the method of Deeley *et al.* (1977) as described by Pemble *et al.* (1986). Poly(A)-containing RNA was isolated as described previously (Taylor *et al.*, 1984).

Construction of a hepatoma cDNA library in λ gt10

Starting from 5 μ g of poly(A)-containing RNA from hepatoma, first strand was synthesized using oligo(dT) primer and AMV reverse transcriptase according to conditions described by Craig *et al.* (1981). Second strand cDNA was synthesized using RNAase H, DNA polymerase I and *E. coli* DNA ligase (NAD⁺) according to conditions described by Gubler & Hoffman (1983). All subsequent enzyme-dependent modifications of double-stranded DNA, described below, were carried out using incubation conditions described by Huynh *et al.* (1985). The cDNA was methylated using *Eco*RI methylase and then made flush-ended by treatment with S₁ nuclease followed by T₄ DNA polymerase. The cDNA (450 ng) was ligated to 1 μ g of phosphorylated *Eco*RI linkers using T₄ DNA ligase and then digested with an excess of *Eco*RI endonuclease. cDNA was separated from contaminating linker fragments by passage through a 1 cm³ Ultrogel AcA34 column equilibrated in 10 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 400 mM-NaCl. After each enzyme incubation the cDNA was purified by sequential extraction, once with phenol/chloroform (1:1, v/v), twice with chloroform and three times with diethyl ether prior to recovery by ethanol precipitation.

Approx. 5 ng of purified cDNA was co-precipitated with 2 μ g of *Eco*RI-cleaved λ gt10 vector DNA and then ligated overnight at 12 °C. Ligated DNA was packaged into phage particles and plated on *E. coli* C600hfl (Maniatis *et al.*, 1982; Huynh *et al.*, 1985). Approx. 5×10^6 recombinants were obtained of which about 30% hybridized to ³²P-labelled hepatoma cDNA.

Screening the cDNA library with an oligonucleotide

Approx. 45000 recombinant plaques from the amplified cDNA library were screened, according to the method of Benton & Davis (1977), with a 17mer oligonucleotide, obtained from Dr. Steve Minter, University of Manchester Institute of Science and Technology, and corresponding to codons specifying amino acids 14–19 from the N-terminus of subunit 7. Washing conditions were as described by Wood *et al.* (1985).

Northern and Southern blotting

Analysis of poly(A)-containing RNA by Northern blotting was carried out as previously described (Taylor *et al.*, 1984). Analysis of endonuclease restriction digests of rat genomic DNA by agarose gel electrophoresis and

Southern blotting was also as described previously (Pemble *et al.*, 1986) with the exception that Hybond-N was used as the transfer membrane. Blotting procedures and washing conditions were those recommended by the manufacturer.

Subcloning and nucleotide sequence analysis

Mini-preparations of plasmid DNA and recombinant phage DNA from plate lysates and all subcloning procedures were as described by Maniatis *et al.* (1982). *Eco*RI-excised inserts of recombinant λ bacteriophage were subcloned into the *Eco*RI site of pUC13. *Eco*RI-excised inserts from these subclones were digested with either *Ava*II or *Hind*III, blunt-ended with the large (Klenow) fragment of *E. coli* DNA polymerase I and ligated in both orientations to *Sma*I-cut M13mp11. The nucleotide sequences were determined by the incorporation of ³⁵S-labelled dCTP using the dideoxy-sequencing method of Sanger *et al.* (1977).

Radiolabelling of probes

Oligonucleotide probes were phosphorylated at the 5' termini by using T₄ DNA polynucleotide kinase and [γ -³²P]dATP as described by Maniatis *et al.* (1982). All other probes were labelled with [α -³²P]dCTP by using the random primer method (Feinberg & Vogelstein, 1983). All probes were routinely labelled to a specific activity of 5×10^8 – 2×10^9 c.p.m./ μ g.

RESULTS AND DISCUSSION

Isolation and characterization of a cDNA clone encoding GSH transferase subunit 7

Starting from mRNA isolated from a DAB-induced hepatoma we have prepared and amplified a large (5×10^5 recombinants) cDNA clone library in λ gt10. Clones complementary to mRNA coding for subunit 7 were selected from 45000 recombinant plaques using a ³²P-labelled, 17 mer oligonucleotide probe which corresponded to amino acid residues 14–19 of subunit 7. Seven positives were plaque purified and the recombinant phage DNA was examined by Southern blot analysis both for hybridization to the oligomeric probe and for cross-hybridization within the cDNA inserts. Five cross-hybridizing clones were subcloned in plasmid pUC13 prior to sequencing in M13 phage (see the Materials and methods section). Of these clones four were judged to be full-length from the presence at one end of an oligo(dT) tail and, at the other end, sequences which encode the N-terminus of the protein (Suguoka *et al.*, 1985). We have determined the complete nucleotide sequence of the longest cDNA insert contained within clone pGSTr7, on both strands, and all restriction sites used were confirmed by overlapping sequence ladders (S. E. Pemble, unpublished work). Excluding the poly(A) tail, pGSTr7 is 738 nucleotides long, containing an open reading frame of 630 nucleotides, and 65 nucleotides 3' non-coding and 43 nucleotides 5' non-coding sequences. No difference in sequence has been observed to occur between pGSTr7 and the other three full-length clones. The nucleotide sequence obtained was virtually identical to that published by Suguoka *et al.* (1985) with two nucleotide changes and with a further four nucleotides of novel 5' non-coding sequence (GTCC). One nucleotide change affects the codon specifying the N-terminal

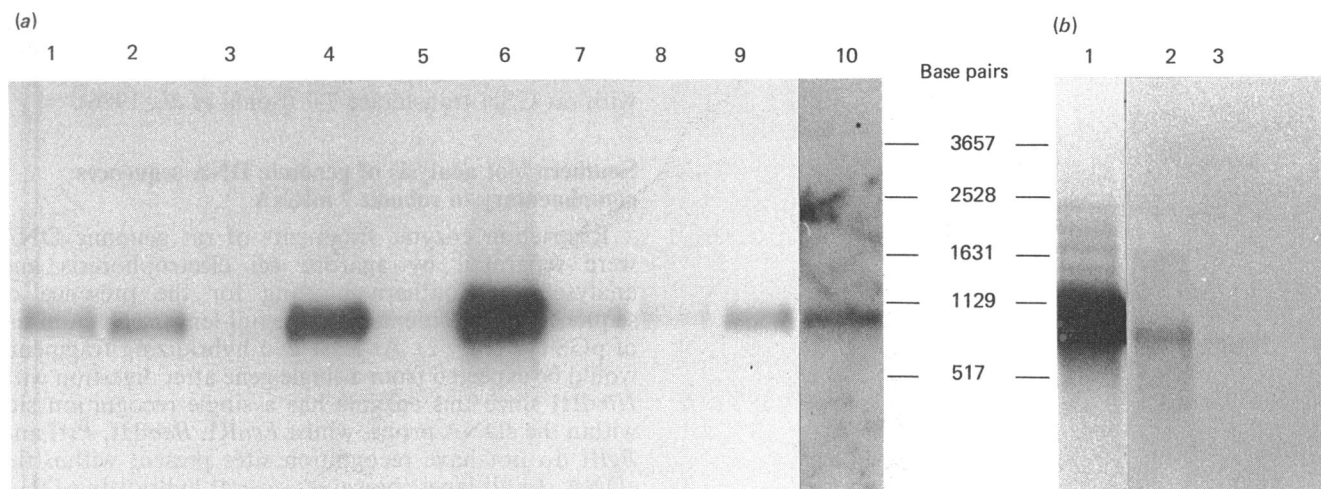


Fig. 1. Tissue distribution of mRNA species hybridizing to cDNA encoding subunit 7

(a) Poly(A)-containing RNA isolated from nine normal rat tissues and hepatoma was analysed under denaturing conditions in 1.1% (w/v) agarose gels. The RNA was transferred to Biodyne A transfer membrane, hybridized with 32 P-labelled pGStr7 and autoradiographed; 8 μ g of poly(A)-containing RNA was loaded in each lane. Tissue of origin was as follows: lane 1, adrenal gland; lane 2, kidney; lane 3, testis; lane 4, epididymis; lane 5, liver; lane 6, hepatoma; lane 7, spleen; lane 8, heart; lane 9, lung; lane 10, skeletal muscle. DNA fragments of known size were linearized pAT153 (3657 base pairs), the products of a *Pst*I/*Bam*HI double digest of pAT153 (2528 and 1129 base pairs) and products of a *Hinf*I digest of pAT153 (1631 and 517 base pairs), treated in the same way as the RNA. (b) Poly(A)-containing RNA isolated from human placenta and lymphocytes were analysed as described in (a). RNA isolated from rat hepatoma was included as a mobility marker; 5 μ g of poly(A)-containing RNA was loaded in each lane. Tissue of origin was as follows: lane 1, rat hepatoma; lane 2, human placenta; lane 3, human lymphocytes. Size markers were as described in (a).

residue (proline, CCG \rightarrow CCA) of the mature protein and the other occurs in the codon for amino acid residue 169 (glycine, GGC \rightarrow GGT) but neither change yields an amino acid substitution. These changes were found in all five clones isolated and are assumed to be a difference associated with the strain of rat from which the hepatoma was taken.

Tissue distribution of GSH transferase mRNA sequences with homology to hepatoma GSH transferase subunit 7 cDNA

Poly(A)-containing RNA isolated from nine normal rat tissues and a DAB-induced primary hepatoma was subjected to agarose gel electrophoresis under denaturing conditions and analysed by Northern blotting using 32 P-labelled cDNA insert of clone pGStr7 as probe. An mRNA species hybridizing to the subunit 7 probe was detected in all the tissues examined (Fig. 1a). This mRNA (approx. 940 nucleotides) was most abundant in the DAB-induced hepatoma (lane 6) but barely detectable in the normal liver (lane 5), a result which is consistent with a previous analysis of mRNA isolated from AAF-induced hepatoma and normal liver (Suguoka *et al.*, 1985). The result is also consistent with GSH transferase 7-7 constituting 25% of the cytosolic GSH transferases in DAB-induced hepatoma but being barely detectable in normal liver (Meyer *et al.*, 1985; Ketterer *et al.*, 1986). This induction has been estimated as greater than 30-fold in both AAF-induced and 3-methyl-DAB-induced hepatoma (Satoh *et al.*, 1985).

However, in common with the differential tissue expression of other subunits of GSH transferases, a polypeptide believed to be subunit 7 has also been reported to occur in kidney, skeletal muscle and placenta

(Meyer *et al.*, 1985; Satoh *et al.*, 1985; Ketterer *et al.*, 1986). Using the hepatoma as reference, hybridizing mRNA species of similar size can be seen, in order of decreasing abundance, in epididymis, kidney, adrenal gland, lung, spleen, skeletal muscle and heart (Fig. 1a, lanes 4, 2, 1, 9, 7, 10 and 8 respectively). The testis (lane 3) is similar to normal liver (lane 5) and contains barely detectable levels of hybridizing mRNA. These results show that amongst the normal rat tissues analysed, the epididymis contains the highest levels of mRNA hybridizing to this probe. In previous studies of normal tissues, the highest levels of GSH transferase 7-7 were observed in the kidney (Meyer *et al.*, 1985; Ketterer *et al.*, 1986). It is noteworthy that we have also observed high levels of several other GSH transferase subunits in the cell-free translation products of epididymal mRNA (Pemble *et al.*, 1986).

Human mRNA sequences with homology to rat hepatoma GSH transferase subunit 7 cDNA

Using 32 P-labelled cDNA insert of pGStr7 as probe, a Northern blot analysis was carried out on poly(A)-containing RNA isolated from human placenta and lymphocytes. mRNA isolated from rat DAB-induced hepatoma was also included on this blot as a mobility marker (Fig. 1b, lane 1). The blot was hybridized under the same conditions as those used for the rat mRNAs, but washed under conditions of reduced stringency ($0.2 \times$ SSC at 37 $^{\circ}$ C instead of $0.2 \times$ SSC at 55 $^{\circ}$ C). A band hybridizing to the subunit 7 probe was detected in human placental mRNA and at very low levels in human lymphocyte mRNA (Fig. 1b, lanes 2 and 3). In the placental poly(A)-containing RNA this hybridizing mRNA is still detectable after washing the blot at the

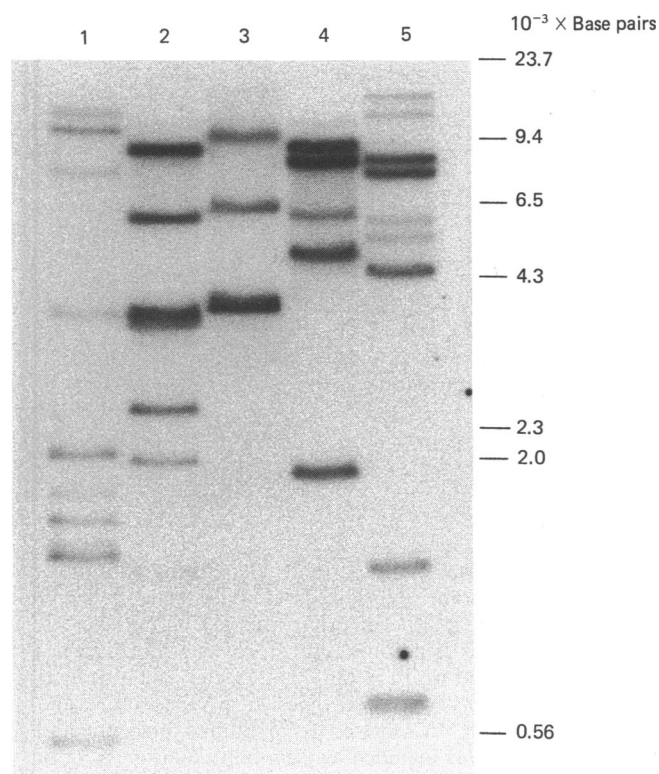


Fig. 2. Southern blot analysis of genomic DNA sequences complementary to cDNA encoding GSH transferase subunit 7

Genomic DNA (20 μ g) isolated from the liver of a single rat was digested to completion with a restriction nuclease as indicated below. The resulting DNA fragments were separated by electrophoresis on a 0.6% agarose gel and transferred to Hybond N blot transfer membrane. The blot was probed with the 32 P-labelled *Eco*RI-excised cDNA insert of pGStr7. This probe contains restriction sites for *Hind*III but not *Bgl*II, *Eco*RI, *Bam*HI and *Pst*I. Hybridizing fragments were visualized by autoradiography. Lanes correspond to genomic DNA digested with: lane 1, *Pst*I; lane 2, *Eco*RI; lane 3, *Bam*HI; lane 4, *Bgl*II; lane 5, *Hind*III. DNA fragments of known size were from the products of a *Hind*III digest of bacteriophage λ (23.7, 9.4, 6.5, 4.3, 2.3, 2.0 and 0.56×10^3 base pairs).

higher stringency (0.2 \times SSC, 55 $^{\circ}$ C) although the signal is considerably weaker (S. E. Pemble, unpublished work). These bands of approx. 900 and 850 nucleotides in length (placenta and lymphocyte respectively) are slightly smaller than that detected in the mRNA isolated from rat tissues. We believe that the hybridizing band in placental mRNA is mRNA coding for the subunit of human GSH transferase π since its *N*-terminal sequence has 20 of the first 22 amino acid residues in common with that of rat subunit 7 (see Ketterer *et al.*, 1986). The weak band in lymphocytes may encode a subunit of either human GSH transferase π or ρ since these two enzymes have great structural similarity (see Mannervik, 1985). In addition, antibody to GSH transferase 7-7 has cross-immunoreactivity with the acidic GSH transferase π from human placenta and GSH transferase ρ from human erythrocytes (Sato *et al.*, 1985). A further parallel with rat GSH transferase 7-7 is that a human

enzyme identified as GSH transferase π has been reported to increase markedly in pre-neoplastic foci and hepatomas and this protein also has common antigenicity with rat GSH transferase 7-7 (Soma *et al.*, 1986).

Southern blot analysis of genomic DNA sequences complementary to subunit 7 mRNA

Restriction enzyme fragments of rat genomic DNA were separated by agarose gel electrophoresis and analysed by Southern blotting for the presence of sequences complementary to the full-length cDNA insert of pGStr7 (Fig. 2). At least two hybridizing fragments would be expected from a single gene after digestion with *Hind*III since this enzyme has a single recognition site within the cDNA probe, whilst *Eco*RI, *Bam*HI, *Pst*I and *Bgl*II do not have recognition sites present within the cDNA. In all lanes, however, several hybridizing DNA fragments of different intensities are visible irrespective of the presence or absence of the appropriate cleavage site within the cDNA probe. A large number of hybridizing fragments would result from a single complementary genomic sequence only if there are intron sequences containing several recognition sites for each of the enzymes. This interpretation is most unlikely since it would require the intron sequences to contain several of the low-frequency cleavage site recognized by each enzyme. The multiple hybridizing restriction fragments are suggestive of a number of genes or pseudogenes complementary to mRNA coding for subunit 7.

In conclusion, we have shown that subunit 7 mRNA, which is dramatically increased in hepatocarcinogenesis, is widely distributed in normal rat tissues and that a highly related mRNA occurs in human placenta. We believe that this cross-hybridization of a rat cDNA probe with a human mRNA will assist in the selection of cDNA clones encoding subunits of human GSH transferases.

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